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Rearing of ectoparasitoid *Diapetimorpha introita* on an artificial diet: supplementation with insect cell line-derived factors

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Abstract. We investigated the use of two insect cell lines to improve an artificial diet (DI) for the pupal ectoparasitoid *Diapetimorpha introita*. DI was supplemented with Grace's culture medium conditioned with IPL-LdFB, a cell line derived from fat body of the gypsy moth, *Lymantria dispar* (FBCell diet), and with Grace's medium conditioned with Sf9, a cell line derived from ovaries of the fall armyworm, *Spodoptera frugiperda* (Sf9Cell diet). The diets were also chemically analyzed for nutrients and any deficiencies were filled by the addition of nutrients. One-half ml aliquots of each diet were encapsulated in paraffin domes and newly hatched larvae of *D. introita* were placed on each diet (one larva/dome) and allowed to develop to the adult stage. Providing fresh diet on day four when the larvae were in the third instar did not improve parasitoid production. Compared with DI, only Sf9Cell had a positive effect on the parasitoid's growth, increasing the size of male parasitoids. The parasitoids, however, took longer to develop to the adult stage than those reared on the natural host. Neither cell line significantly enhanced the average weight of female parasitoids, shortened developmental time, nor increased % cocoons produced and % adult emergence. Providing additional nutrients (amino acids, vitamins, cations and anions, fatty acids and milk/egg protein) to both diets (based on chemical analyses of the cell line-supplemented diets) enhanced the average weight of the females on Sf9Cell and males and females on FBCell. The nutritional additions, however, did not improve the developmental time, pupation and adult emergence.

Key words: Hymenoptera, parasitoid, *Spodoptera*

Introduction

Diapetimorpha introita (Cresson) (Hymenoptera: Ichneumonidae) is a native pupal ectoparasitoid of the USA that attacks overwintering armyworms, *Spodoptera frugiperda*, (J. E. Smith) and *S. exigua* (Hübner) (Pair and Gross, 1984, 1989). *D. introita* has been proposed as a key agent in IPM programs

of armyworms. The parasitoid has potential by augmentative releases in reducing low overwintering populations of *Spodoptera* spp. before they emerge (Pair, 1995). However, cost-effective augmentative releases of the parasitoid cannot be made because the parasitoid must be reared on the natural host. To address this need, Carpenter and Greany (1998) developed an artificial diet (free of insect components) for rearing *D. introita*. Although *D. introita* could be reared on this diet to the adult stage, the parasitoids had longer developmental times, were smaller in size and had reduced fecundity compared to wasps reared on host pupae. In fact, many artificial diets that have been developed for parasitoids are not optimal with regard to the quality of insects produced (Grenier et al., 1994). In many cases, the diet can be improved by adding insect materials such as hemolymph back to the diet (Nettles, 1990). Because of the labor associated with collecting hemolymph and other problems such as melanization, substitute materials that can be obtained economically and added directly to the diets must be identified before major advances in artificial culture of insect parasitoids can be achieved (Grenier et al., 1994). One approach that has been tested is the use of insect cell line media. Rotundo et al. (1988) reared a small number of the aphid endoparasitoid, *Lysiphlebus fabarum* (Marshall) from first instar larva to adult stage *in vitro*, using a cell line derived from the dipteran *Ceratitis capitata* Wiedeman. Ferkovich et al. (1994) showed that several insect cell lines stimulated embryonic development *in vitro* of *Microplitis croceipes*, an endoparasitoid of *Heliothis* spp. Conceivably, other growth regulatory molecules required for growth and reproduction of parasitoids may be produced by insect cell lines derived from the host insect. Since the technology for mass culture of insect cell lines has been developed commercially for production of baculoviruses and recombinant proteins (Maiorella et al., 1988; Cho et al., 1989; Rice et al., 1993), this same technology could supposedly be used to mass culture cells for fortifying the artificial diets of *D. introita* and other parasitoids.

In this study, we investigated the potential of using two insect cell lines, one derived from the ovaries of *S. frugiperda,* a natural host of *D. introita* and one derived from fat bodies of the gypsy moth, *Lymantria dispar* (L.) to promote growth and development of *D. introita.*

Materials and methods

Insect rearing

Spodoptera frugiperda (J. E. Smith) and *D. introita* (Cresson) were reared according to Carpenter and Greany (1998). We received eggs of *D. introita* by

mail from the Insect Biology and Population Management Research Laboratory, ARS, USDA, Tifton, GA. The eggs were placed individually in cells of a 96 well Falcon® tissue culture plate at 21±1 °C and 70% R. H. First instars of *D. introita* that hatched overnight (12 h period) were used in the bioassay. The larvae were held at 29±1 °C during the bioassay.

Diet preparation and encapsulation of diet

Both cell-line supplemented diets were prepared under aseptic conditions in a clean room. The clean room (1.5 m × 2.4 m ×2.4 m) contained two HEPA filter modules (Class 100 air, 700 cfm; Clesta Cleanroom, North Syracuse, NY). Prior to using the room, an ozonator (model XL-15, Living Air, Blaine, MN) was turned on the lowest setting for ozone output of 66 mg/h in the room for 12 h to decontaminate it.

Original artificial diet

The original artificial diet (DI) containing ground beef liver, chicken egg yolk, and the amino acid L-glutamine (Sigma, St. Louis, MO) was prepared according to Carpenter and Greany (1998). All the ingredients were added to 100 ml of serum-free SF-900 II cell culture medium. The diet was encapsulated in Parafilm® using a diet encapsulation apparatus (Greany and Carpenter, 1996). Diet was dispensed at 0.5 ml of diet/dome with 24 domes/sheet. Each diet sheet was covered with a modified (bottomless) Falcon® tissue culture plate (Sigma, St. Louis, MO) so that each dome (one larva/dome) was situated within a well. The entire culture plate was covered with a Plexiglas® plate to prevent escape of the larvae.

Preparation of cell line-supplemented diets

The cell lines were originally derived from different tissues and species and each line was cultured in Grace's medium (Sigma, St. Louis, MO) with different additives (Grace, 1962). IPLB-LdFB was derived from fourth and fifth instar larval fat body of *L. dispar* and maintained in Grace's medium supplemented with 10% fetal bovine serum (FBS) and 1% bovine serum albumin (BSA) as described by Lynn et al. (1988). The Sf9 cell line was an embryonic line originally derived from ovaries of the fall armyworm, *S. frugiperda*, and purchased from ATCC, Rockville, MD. The cells were cultured in Grace's medium with 10% FBS, 1% BSA and 0.33% lactalbumin enzymatic hydrolysate (Sigma, St. Louis, MO). For larger-scale culture of the cell lines, cells were grown in 250 ml magnetic spinner flasks (Bellco Glass, Vineland, NJ) at 29 °C. Both cell lines were grown to densities of 1.3×10^5

to 2×10^5 cells/ml 10 days post planting. For the experiments, 25 ml of cell suspension were centrifuged at $250 \times g$ for 2 min at room temperature. The supernatant then was substituted for the SF-900-II medium in preparing the artificial treatments diets.

Nutritional analyses

Amino acid analyses

A 300 µl sample of each diet and pupae were singly placed in a 2 ml centrifuge plastic vial. The vials with the samples were centrifuged for 10 min at 16,000 *g* (crude sample). Ten µl of each sample were transferred to a clean centrifuge vial and diluted with 0.1 N hydrochloric acid to reach a final concentration of 1:100; then samples were reacted with FMOC (9-fluorenylmethyl chloroformate, Sigma F-0378), and analyzed using the modified high performance liquid chromatography (HPLC) method reported by Rojas et al. (1998).

Water soluble vitamins, cations, and anions

One hundred g of each crude sample were transferred to a clean centrifuge tube and diluted with 18 megaohm Milli-Q water to reach a final concentration of 1:10 for water soluble vitamins, and 1:100 for cations and anions. Each sample was analyzed by liquid chromatography using the procedures included in the DIONEXR system (DX 500, Houston, TX).

Fatty acids

Two hundred mg of each sample were extracted, reacted and analyzed by gas chromatography (GC) using the modified H. Williams (TAMU-Entomology Dept., College Station, TX, USA, 1997, personal communication) method. Extracts were made from 2:1 methanol: chloroform, from 3 consecutive washes with distilled water each. The samples were concentrated to an oil, heat dried, and reacted with BF3/MEOH (Boron trifluoride/methanol, 14% w/v, Alltech Associates, Cat. #18041) to form methyl esters. The reacted samples were dissolved in hexane, washed with distilled water, concentrated with nitrogen, and analyzed using a Hewlett-Packard GC system (Thompson, 1979).

Total protein

The total protein content in each sample was determined using Bio-Rad (detergent compatible) protein assay (Bio-Rad kit 500-0116) with bovine serum albumin (Pierce No. 23209) as standard. Absorbance was measured in a Bausch & Lomb Spectronics 88 spectrometer.

Replications per analysis
For each analysis, a mean was calculated from three repetitions per each sample.

Nutritional balancing of cell line-supplemented diets

Based on chemical analyses of both cell line-supplemented diets and host pupae, any deficiencies were balanced to the level of that found in host pupae. No attempt was made to reduce the level of the nutrients in the diets that were in excess of those found in host pupae. The chemicals were added directly to 25 ml of cell conditioned media prior to adding the beef liver and egg yolk. The following insect cell culture tested chemicals were used in balancing the diets and all were purchased from Sigma, St. Louis, MO, unless otherwise indicated: amino acids: histidine, L-asparagine, L-arginine, L-glutamine, L-glutamic acid, L-threonine, L-alanine, L-proline, L-methionine, L-valine, L-phenylalanine, L-isoleucine, L-leucine, L-lysine, L-tyrosine; vitamins: L-ascorbic acid, pyridoxine, thiamine, niacin amide, riboflavin, vitamin B_{12}; cations: ammonium chloride, potassium phosphate (monobasic), magnesium chloride; anions: sodium fluoride, and potassium phosphate; protein: milk and egg protein (85% powder, M-L-O Products, Fairfield, CA). Except for oleic acid (Sigma, St. Louis, MO), no additional supplementation of fatty acids to the two cell line supplemented diets was made since the fatty acids were already fairly well balanced in both cell line supplemented diets.

Treatment diets

(1) *host pupae*; (2) *DI*-original artificial diet; (3) *FBControl*$_A$-DI prepared with Grace's medium; (4) *FBControl*$_B$-DI prepared with Grace's medium with 1% BSA and 10% FBS; (5) *FBCell*-DI prepared with IPL-LdFB cell-conditioned Grace's medium with 1% BSA and 10% FBS); (6) *Sf9Control*-DI prepared with Grace's medium with 10% FBS, 1% BSA, and 0.33% lactalbumin enzymatic hydrolysate; (7) *Sf9Cell*-DI prepared with S9 cell-conditioned Grace's medium with 10% FBS, 1% BSA and 0.33% lactalbumin hydrolysate; (8) *NS-FBCell*-DI prepared with IPL-LdFB cell-conditioned Grace's medium with 1% BSA and 10% FBS and nutritionally supplemented; *NS-Sf9Cell*-DI prepared with S9 cell-conditioned Grace's medium with 10% FBS, 1% BSA and 0.33% lactalbumin hydrolysate and nutritionally supplemented; (9) P-FBCell-DI prepared with IPL-LdFB cell-conditioned Grace's medium with 1% BSA and 10% FBS and nutritionally supplemented only with milk and egg protein powder; and (10) P-Sf9Cell-DI prepared with S9 cell-conditioned Grace's medium with 1% BSA and 10% FBS and 0.33%

lactalbumin hydrolysate and nutritionally supplemented only with milk and egg protein powder. The composition of the various diets is given in Table 1.

First test: Evaluation of a fat body-derived cell line and two cell culture supplements
In the first test, the objective was to determine the potential growth enhancing effects of a fat body-derived cell line, IPL-LdFB, and also two supplements, BSA and FBS, that were used in culturing the cells. The following treatments were run: DI, $FBControl_A$, $FBControl_B$ and FBCell. Fresh diet was not provided during the experiment.

Second test: Comparison of the fat body-derived cell line with an embryonic-derived cell line
In the second test, the objective was to compare the embryonic cell line, Sf9, with the fat body cell line, IPLB-LdFB. Encapsulated diet was replaced with fresh diet four days after the neonates were placed on the diets. The treatments were: host pupae, DI; $FBControl_B$, FBCell, Sf9Control, and Sf9Cell.

Third test: Chemical analyses of FBCell and SF9Cell diets and correction of nutritional deficiencies
In the third test, the FBCell and Sf9Cell diets were chemically analyzed for nutritional composition and an attempt was made to correct any nutritional deficiencies relative to the levels in host pupae (Table 1 in Addendum). Diet was replaced once four days after neonates were placed on the diets as in the second experiment. In this experiment the $FBConrol_A$ and Sf9Control diets were not run; the following treatments were run: host pupae, DI; NS-FBCell; and NS-Sf9Cell.

Fourth test: Protein supplementation of FBCell and Sf9Cell diets
In the fourth test, based on the protein analyses of the FBCell and Sf9 diets, both were supplemented only with the milk and egg protein powder. The treatments run in this experiment were: host, DI, P-FBCell and P-Sf9Cell. This test was run simultaneously with the second test and therefore, the same control treatments, DI diet and host, were used in the analysis of data.

Bioassay

First instar larvae that hatched within a 12-hour period were placed on encapsulated diet domes (one larva/dome) in individual cells of a 24 well plate to feed and develop to adults. The larvae were allowed to feed and develop to adults (described below) at 29±1 °C and 70% RH. The third instar larvae were transferred to the new diet domes using a camel hair brush. As the adults

Table 1. Ingredients of diets used to rear *D. intriota*

Components	Diets									
	DI	FB Control$_A$	FB Control$_B$	FB Cell	S9 Control	S9 Cell	NS-FB[a] Cell	NS-Sf9[a] Cell	P-FB Cell	P-SF9 Cell
Cell culture media (ml)										
SF-900-II	100 ml									
Grace's		100 ml	100 ml		100 ml					
Cell-conditioned media (ml)										
IPL-LD FB-Grace's				100 ml			100 ml		100 ml	
Sf9-Grace's						100 ml		100 ml		100 ml
Basic ingredients (g/100 ml)										
Ground beef liver	28.5 g	28.5 g	28.5 g	28.5 g	28.5 g		28.5 g	28.5 g	28.5 g	28.5 g
Chicken egg yolk	26.0 g	26.0 g	26.0 g	26.0 g	26.0 g		26.0 g	26.0 g	26.0 g	26.0 g
L-glutamine	0.8 g	0.8 g	0.8 g	0.8 g	0.8 g		0.8 g	0.8 g	0.8 g	0.8 g
Proteins (g/100 ml)										
Bovine serum albumin			1.0 g	1.0 g	1.0 g	1.0 g	1.0 g	1.0 g	1.0 g	1.0 g
Fetal bovine serum			10.0 g	10.0 g	10.0 g	10.0 g	10.0 g	10.0 g	10.0 g	10.0 g
Lactalbumin hydroysate (peptone)					0.33 g	0.33 g		0.33 g		0.33 g
Milk/egg protein									4.7 g	5.0 g

[a] Diet was nutritionally supplemented to fill chemical deficiencies shown by comparison of chemical analyses of FB Cell and S9 Cell diets with host pupae in Table 1 of Addendum.

Table 1. (Continuation of NS-FB Cell[a] and NS-Sf9 Cell[a] diets)

	NS-FB Cell	NS-Sf9 Cell
Amino acids (μg/ml)		
Histidine	944.70	944.70
Asparagine	94.50	94.50
Arginine	618.26	618.26
Glutamine	2626.77	2626.77
Asp + sec	674.44	694.48
Glutamic acid	369.84	991.43
Threonine	985.59	985.59
Glycine	414.84	588.46
Alanine	2206.19	2206.19
Proline	705.60	705.60
Nethionine	497.02	497.02
Valine	605.80	605.80
Phenylalanine	518.54	518.54
Isoleucine	1340.13	1340.13
Leucine	796.57	1371.22
Lycine	2032.31	2032.31
Tryrosine	919.07	1259.33
Vitamins (μg/ml)		
Ascorbic acid	48.00	48.00
Pyridoxine HCL	178.67	178.67
Thiamine	23.00	23.00
Niacin amide	27.00	27.00
Folic acid	0.00	0.00
Riboflavin	1.73	75.00
B12	8.33	8.33
Cations (μg/ml)		
Lithium	0.19	0.00
Sodium	11.08	69.86
Ammonium	20.09	28.44
Potassium	219.91	399.73
Magnesium	51.39	51.39
Calcium	10.24	9.73
Anions (μg/ml)		
Fluoride	21.49	23.33
Chloride	464.86	350.93
Phosphate	830.43	830.43
Sulfate	84.19	74.32
% Fatty acids		
Palmitoleic	0.170	0.502
Palmitic	0.956	4.264
Linolenic	0.289	2.428
Linoleic	0.587	6.525
Oleic	0.242	0.242
Stearic	0.821	0.312

[a] Diet was nutritionally supplemented to fill chemical deficiencies shown by comparison of chemical analyses of FB Cell and S9 Cell diets with host pupae in Table 1 of Addendum.

emerged, they were held individually in plastic portion cups (102 ml) with a source of undiluted honey for 24 h before they were weighed.

There were six replications per treatment in the first and second tests and three replications per treatment in the third and fourth tests. A 24 well plate containing 24 larvae on a diet constituted one replication. The number of days required to develop to the adult stage, and the percentage of cocoons and adults produced were recorded. The percentage of adults produced was calculated from the total number of 24 larvae originally set on the diet per replication.

Statistical analysis

The effects of the various diets on parasitoid growth and development were subjected to two-way analysis of variance (ANOVA) using the Statistical Analysis System (SAS, 1989). Data from each of the four experiments were analyzed separately. Percent data was transformed using square root transformation and arc sine transformation. Separation of means was done using the Duncan procedure.

Results

First test

In the first test, the parasitoids were not provided fresh diet and only the IPL-LdFB cell line was tested (Table 1). Neither the cell line conditioned medium (FBCell) nor the two control diets, FBControl^{A} and FBControl^{B} significantly improved the developmental time of male or female parasitoids (♀, $F = 1.35$, $df = 3$, $p = 0.297$; ♂, $F = 1.41$, $df = 3$, $p = 0.286$), percentage of cocoons produced ($F = 1.86$, $df = 3$, $p = 0.177$), emergence of adult males and females ($F = 0.89$, $df = 3$, $p = 0.466$) or average weights of males and females (♂, $F = 2.89$, $df = 3$, $p = 0.075$; ♀, $F = 2.29$, $df = 3$, $p = 0.124$).

Second test

In the second test, two cell lines were evaluated. The F-value was significant for male and female development (♂, $F = 3.05$, $df = 5$, $p = 0.03$; ♀, $F = 4.33$, $df = 5$, $p = 0.007$), adult emergence ($F = 4.22$, $df = 5$, $p = 0.008$) and the average weight of male and female weights (♂, $F = 5.04$, $df = 5, 21$, $p = 0.003$; ♀, $F = 2.58$, $df = 5, 21$, $p = 0.05$), but was not significant for cocoon production ($F = 1.51$, $df = 5$, $p = 0.22$) (Table 2a). Duncan's multiple range test indicated that neither of the cell line-supplemented diets were significantly different

Table 2a. Growth and developmental attributes of *D. introita* reared on DI, two control diets, and a cell line-supplemented diet

Treatment diets	Developmental time (days) (means ± SE)		% Cocoons produced (means ± SE)	% Emergence (means ± SE)	Adult wt. (mg) (means ± SE)	
	Male	Female			Male	Female
Test 1[a]						
DI	20.2 0.7	21.1 + 1.7	68.1 ± 11.1	51.0 ± 10.9	20.2 ± 1.3	31.2 ± 0.9
$FBControl_A$	19.5 ± 0.2	24.7 ± 1.2	67.8 ± 10.1	43.9 ± 7.7	25.8 ± 2.4	36.0 ± 4.5
$FBControl_B$	20.6 ± 0.5	22.1 ± 1.0	69.9 ± 12.9	45.0 ± 7.3	26.9 ± 2.2	41.6 ± 6.2
FBCell	18.7 ± 0.4	23.0 ± 1.1	83.8 ± 12.5	36.5 ± 6.5	27.2 ± 2.2	43.2 ± 4.9

[a] None of the F-values were significant at $p = 0.05$.

Table 2b. Growth and developmental attributes of *D. introita* reared on DI supplemented with conditioned media from two insect cell lines without additional nutrients (Test 2) and with additional nutrients added (Test 3)

Treatment diets[a]	Developmental time (days) (means ± SE)		% Cocoons produced (means ± SE)	% Emergence (means ± SE)	Adult wt. (mg) (means ± SE)	
	Male	Female			Male	Female
Test 2						
Host	19.1 ± 0.8 c	18.4 ± 0.9 b	73.1 ± 5.0 a	46.4 ± 4.8 a	25.0 ± 1.8 bc	43.0 ± 2.1 a
DI	19.9 ± 0.7 bc	22.2 ± 0.8 a	67.9 ± 4.8 a	46.5 ± 4.4 a	21.8 ± 1.6 c	34.5 ± 1.9 b
$FBControl_B$	21.7 ± 0.8 ab	21.8 ± 1.0 a	53.1 ± 5.5 a	23.2 ± 5.2 b	25.6 ± 1.9 bc	39.7 ± 2.2 ab
FBCell	20.4 ± 1.2 abc	20.8 ± 1.4 ab	69.4 ± 7.8 a	20.9 ± 7.3 b	22.9 ± 2.7 c	38.6 ± 3.1 ab
Sf9Control	21.5 ± 0.5 abc	23.5 ± 1.0 a	60.8 ± 6.0 a	28.4 ± 5.7 ab	28.2 ± 2.1 ab	39.7 ± 2.4 ab
Sf9Cell	22.6 ± 0.9 a	24.0 ± 1.0 a	68.5 ± 6.0 a	38.1 ± 5.7 ab	31.5 ± 2.1 a	40.1 ± 2.4 ab
Test 3						
Host	16.1 ± 0.4 b	16.0 ± 0.1 b	64.2 ± 7.0 a	50.8 ± 7.0 a	21.6 ± 0.9 b	36.6 ± 0.8b
DI	18.5 ± 2.5 ab	19.8 ± 0.2 a	51.9 ± 5.5 a	37.4 ± 1.9 a	19.1 ± 0.5 b	28.9 ± 2.2 c
NS-FBCell	18.5 ± 0.9 ab	19.0 ± 1.4 a	52.8 ± 7.4 a	38.9 ± 7.4 a	22.2 ± 0.5 ab	41.1 ± 2.9 ab
NS-Sf9Cell	19.4 ± 1.4 a	20.4 ± 1.3 a	59.1 ± 8.9 a	34.4 ± 11.8 a	25.1 ± 1.3 a	45.1 ± 2.6 a

[a] Means followed by the same letter in each column are not significantly different ($p < 0.05$).

from their respective control diets ($FBControl_B$ vs. FBCell and Sf9Control vs. Sf9Cell) for male and female developmental time, % cocoons produced, % emergence, or average adult weight. However, males and females reared on the FBCell diet developed at the same rate as host-reared parasitoids. Similarly, males reared on the Sf9Cell diet did not take longer to develop than males reared on the host or DI diet. Also, Sf9Control reared-males were larger than DI diet-reared males and Sf9Cell-males were larger than males reared on the host and DI diet.

Third test

In the third test, the two cell line supplemented diets were additionally fortified with nutrients based on the chemical analyses of the diets (Addendum, Table 1). The F-values were significant for female development (♀, F = 5.72, df = 3, p = 0.009), and male and female average weight (♂, F = 6.99, df = 3, p = 0.004; ♀, F = 11.73, df = 3, p = 0.0004) and not significant for male development (♂, F = 2.9, df = 3, p = 0.07), cocoon production (F = 0.64, df = 3, p = 0.60) and emergence (F = 0.90, df = 3, p = 0.47) (Table 2b). Duncan's multiple range test indicated that mean developmental times of females were significantly longer on the DI diet and NS-FBCell And NS-Sf9Cell diets than on the host, but there were no differences between the DI diet and the two nutritionally supplemented diets. The average weight of males reared on the NS-Sf9Cell diet was higher than that of males reared on DI diet or host, but not higher than that of males reared on the NS-FBCell diet. Similarly, females reared on the NS-Sf9Cell diet weighed more than females on the DI diet or host but were not significantly heavier than the females on the NS-FBCell diet.

Fourth test

In the fourth test, both cell line-conditioned diets which were deficient in protein content relative to host pupae (Addendum, Table 1), were supplemented with the milk and egg protein powder to approximate that of the host pupae. The F-values were significant for male and female development (♂, F = 5.08, df = 3, p = 0.01; ♀, F = 4.03, df = 3, p = 0.03), and male and female average weight (♂, F = 9.57, df = 3, p = 0.001; ♀, F = 4.01, df = 3, p = 0.03) and not significant for cocoon production (F = 2.44, df = 3, p = 0.11) and emergence (F = 1.09, df = 3, p = 0.39) (Table 3). Duncan's multiple range test indicated that males and females reared on the P-FBCell diet took longer to develop than those reared on the host, but parasitoids reared on the P-Sf9Cell diet developed in the same amount of time. Males reared on the P-FBCell diet had long and enlarged abdomens and were significantly heavier than males reared on the P-Sf9Cell diet or the DI diet or host. Females reared on the P-FBCell diet also had enlarged abdomens and weighed almost twice as much as females on the DI diet but they were not significantly larger than the host reared females. Females in the 50–60 mg range were never obtained on the DI diet or host in any of the four tests of this study.

Table 3. Growth and developmental attributes of *D. introita* reared on DI supplemented with conditioned media from two cell lines and milk and egg protein (Test 4)

Treatment diets[a]	Developmental time (days) (means ± SE)		% Cocoons produced (means ± SE)	% Emergence (means ± SE)	Adult wt. (mg) (means ± SE)	
	Male	Female			Male	Female
			Test 4			
Host	19.1 ± 0.8 c	18.4 ± 0.9 b	73.1 ± 5.0 a	46.4 ± 5.8 a	25.0 ± 1.8 b	43.0 ± 3.5 ab
DI	19.9 ± 0.7 ab	22.2 ± 0.8 a	67.9 ± 4.8 a	46.5 ± 5.4 a	21.8 ± 1.7 b	34.5 ± 3.3 b
P-FBCell	21.2 ± 1.2 a	22.5 ± 1.0 a	26.2 ± 8.6 a	26.2 ± 10.9 a	36.7 ± 3.3 a	60.4 ± 6.6 a
P-Sf9Cell	18.5 ± 1.2 bc	20.2 ± 1.0 ab	35.4 ± 8.6 a	35.4 ± 10.9 a	24.5 ± 3.3 b	38.1 ± 6.6 ab

[a] Means followed by the same letter in each column are not significantly different ($p > 0.05$).

Discussion

To date, the use of insect cell lines for *in vitro* culture of insects has been reported in two species of hymenopteran endoparasitoids. Rotundo et al. (1988) reared the aphid endoparasitoid, *Lysiphlebus fabarum* (Marshall) from first instar larva to adult stage *in vitro*, using a cell line derived from the dipteran *Ceratitis capitata* Wiedeman. Partial success was achieved in rearing an endoparasitoid, *Microplitis croceipes* from egg through the first instar *in vitro* using insect cell lines (Ferkovich et al., 1991). One of the cell lines, IPL-Ld FB, was found to produce two active factors(s), a dialyzable material (<10 K) that induced germ-band development, and a nondialyzable (>10 K) material that stimulated egg hatch in *M. croceipes* (Ferkovich and Oberlander, 1991).

Endoparasitoids differ from ectoparasitoids in that they develop in a milieu of hemolymph in the host, must orchestrate their development with the physiological changes of the host, and may also require specific host factors (Vinson and Iwantsch, 1980; Greany et al., 1988, 1990; Ferkovich et al., 1991; Grenier et al., 1994). Conceptually, ectoparasitoids generally do not rely on an intricate physiological interaction with their host during their development. Therefore, we surmised that formulating an optimal diet for *D. introita* might be less difficult than for *M. croceipes* (Vinson and Iwantsch, 1980). Since many of the artificial diets that have been developed can be improved by adding insect components, we initially investigated the potential of using the IPL-LdFB cell line to improve the diet for *D. introita* and subsequently evaluated Sf9, a cell line derived from embryonic tissue originating from the host of *D. introita*.

In the first test, we speculated that the IPL-LdFB cell line derived from fat body would provide growth promoting factors for *D. introita* and were

surprised that the $FBControl_B$ diet which contained FBS or the FBCell diet did not significantly affect growth and development of *D. introita*. FBS is a complex supplement that is a rich source of growth factors, carrier proteins, cell protective agents, attachment factors and nutrients (Cartwright and Shaw, 1994) which has been used as a replacement for heat-treated hemolymph in establishing insect cell lines (Trager, 1935; Wyatt, 1956; Grace, 1962) and in formulating artificial diets for parasitoids and predators (Pennacchio et al., 1992; Mitsuhashi and Oshiki, 1993; Obayashi et al., 1994; Watanabe and Mitsuhashi, 1995; Ha et al., 1996; Yamamoto et al., 1997). In addition to containing FBS, the FBCell diet also contained medium conditioned with the IPL-LdFB cell line which we earlier stimulated egg development of the endoparasitoid, *Microplitis croceipes in vitro* (Ferkovich et al., 1991). It is possible that the nutritional content of the diet decays over time; however, replacing the diet with fresh diet 4 days after the neonates were initially placed on the diet (Test 2) did not significantly improve the overall parasitoid qualities measured with the FBCell vs DI diet treatments (Test 1 vs. Test 2A). The possibility also exists that the IPL-LdFB cell line may only produce embryotropic factors for parasitoid development, as in the case of *M. croceipes* (Ferkovich et al., 1991) and does not provide factors capable of promoting larval development required by *D. introita.*

In the second test, we hypothesized that a cell line such as Sf9 that is derived from the natural host of the parasitoid, *S. frugiperida* would promote better growth and development of the parasitoid than a cell line originating from a non-host species such as the IPL-LdFB cell line (used in the FBCell diet) derived from the gypsy moth, *Lymantria dispar* (non-host). We do not have a good explanation why the Sf9 ovarial cell line only enhanced the size of male parasitoids in Test 2B. This enhancement of male size was not due to the products synthesized by the cell line and released into the medium since the Sf9Control diet also significantly produced larger males. The growth promoting effects of the Sf9 diet were probably due to one of the additives used in cell culture such as lactoalbumin hydrolysate, yeastolate or fetal bovine serum. In the first test, FBS did not significantly affect growth of *D. introita* so it seems unlikely that it is responsible for the growth stimulating effect observed in this test.

In the third test the FBCell diet and the Sf9 diet were chemically analyzed to determined if the diets were still deficient in nutrients because of the overall lackluster improvement of the DI diet using the two cell lines in the earlier tests. Fortifying the two diets with certain amino acids, vitamins, cations, anions, and fatty acids did not improve the diets in terms of shortening developmental time and enhancing the rate of emergence although the Sf9Cell diet did produce larger adult male and female parasitoids (Test 2B).

In the fourth test, supplementing the cell-conditioned diets with the milk/egg protein promoted larger males in the case of the FBCell diet but did not have a generally beneficial effect on all the other growth parameters measured. Strand and Vinson (1985) reported bloated larvae and adults in *Trichogramma pretiosum* reared *in vitro* but overcame the problem by limiting the amount of medium in order to prevent overfeeding. Similarly, Grenier et al. (1995) also reported that bloated larvae and pupae with enlarged abdomens and a low protein content were unable to produce adults. In this study, dissection of adult males and females of *D. introita* revealed haemocoels packed with fat bodies. It appears that understanding what metabolic pathways are in play will require further information.

In conclusion, it appears that neither of the cell lines cultured in Grace's culture medium has potential as a source of requisite host factors for optimizing overall growth and development of *D. introita*. However, it should be pointed out that the two cell lines could potentially produce growth promoting products required by *D. introita* when the cells cultured under different conditions such as in other cell culture media with various additives. For example, Ferkovich et al. (1994) reported that the optimal conditions for promoting development of *M. croceipes in vitro* was dependent on the composition of the cell culture medium, such as the type of basal medium, and the species and tissue type of the cell line source; the authors stated that all these factors must be evaluated interactively. In this study, we used Grace's medium because both of the cell lines were already adapted to being cultured in this medium. Also, it is possible that using cell lines from other species and tissue sources and/or additional types of cell culture media and additives could induce the cell lines to produce factors required by *D. introita* for growth and development. These studies suggest that future research should include additional cell lines, cell culture media and various additives for evaluation as well as a study of host specific material (e.g. parasitoid growth factor from host pupae) that may be required such as that reported in the endoparasitoid, *M. croceipes* (Greany et al., 1988, 1990; Ferkovich et al., 1991).

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References

Carpenter, J.E. and P. Greany, 1998. Comparative development and performance of artificially reared vs. host-reared *Diapetimorpha introita* (Cresson) (Hymenoptera: Ichneumonidae) wasps. *Biol. Control* 11: 203–208.

Cartwright, T. and G.P. Shah, 1994. Culture media. In: J.M. Davis (ed), *Basic Cell Culture – A Practical Approach*. IRL Press at Oxford University Press, Oxford. pp. 57–91.

Cho, T., M.L. Shuler and R.R. Granados, 1989. Current developments in new media and cell culture systems for the large-scale production of insect cells. In: *Advances In Cell Culture*, Vol. 7. Academic Press, San Diego. pp. 261–276.

Ferkovich, S.M., C. Dillard and H. Oberlander, 1991. Stimulation of embryonic development in *Microplitis croceipes* (Braconidae) in cell culture media preconditioned with a fat body cell line derived from a nonpermissive host, Gypsy moth, *Lymantria dispar*. *Arch. Insect Biochem. Physiol.* 18: 169–175.

Ferkovich, S.M. and H. Oberlander, 1991. Stimulation of endoparasitoid egg development by a fat body cell line: activity and characterization of factors that induce germ band formation and hatching. In: *Proc. VIII Internat. Conf. Invert. and Fish Tiss. Culture, Tissue Culture Assoc.*, Columbia, USA. pp. 181–187.

Ferkovich, S.M., H. Oberlander, C. Dillard and E. Leach, 1994. Embryonic development of an endoparasitoid, *Microplitis croceipes* (Hymenoptera: Braconidae) in cell line-conditioned media. *In Vitro Cell Dev. Biol.* 30A: 279–282.

Grace, T.D.C., 1962. Establishment of four strains of cells from insect tissue grown *in vitro*. *Nature* 195: 788–789.

Greany, P.D., W.R. Clark and S.M. Ferkovich, 1988. Influence of a host hemolymph protein in stimulating early egg development in *Microplitis croceipes*. *Proc. XVIII Int. Congress Entomol.*, Vancouver, Canada, July 3–9.

Greany, P., W. Clark, S.M. Ferkovich, J. Law and R. Ryan, 1990. Isolation and characterization of a host hemolymph protein required for development of the eggs of the endoparasite *Microplitis croceipes*. In: H.H. Hagedorn, J.G. Hildebrandi, M.G. Caldwell and J.H. Law (eds), *Molecular Insect Science*. Plenum Press, New York and London. p. 306.

Greany, P. and J.E. Carpenter, 1996. Culture medium for parasitic and predaceous insects. U.S. Patent 08/692,565: Docket No. 000010.96. (Application pending).

Grenier, S., P.D. Greany and A.C. Cohen, 1994. Potential for mass release of insect parasitoids and predators through development of artificial culture techniques. In: D. Rosen, F.D. Bennett and J.L. Capinera (eds), *Pest Management in the Subtropics: Biological Control – a Florida Perspective*. Intercept Publishers, Andover, Hampshire, England. pp. 181–205.

Grenier, S., H. Yang, J. Guillaud and L. Chapelle, 1995. Comparative development and biochemical analyses of *Trichogramma* (Hymenoptera: Trichogrammatidae) grown in artificial media with hemolymph or devoid of insect components. *Comp. Biochem. Physiol.* 111B: 83–90.

Ha, S.H., T.H. Park and S.E. Kim, 1996. Silkworm hemolymph as a substitute for fetal bovine serum in insect cell culture. *Biotech. Techniques* 10: 401–406.

Lynn, D.E., E.M. Dougherty, J.T. McClintock and M. Loeb, 1988. Development of cell lines from various tissues of Lepidoptera. In: Y. Kuroda, E. Kurstak and K. Maramorosch (eds), *Invertebrate and Fish Tissue Culture*. Springer-Verlag, New York. pp. 239–242.

Maiorella, B., D. Inlow, A. Shauger and D. Harano, 1988. Large-scale insect cell-culture for recombinant protein production. *Biotechnology* 6: 1406–1408.

Mitsuhashi, J. and T. Oshiki, 1993. Preliminary attempts to rear an endoparasitic fly, *Exorista sorbillans* (Diptera, Tachinidae) *in vitro*. *Japanese Jour. Entom.* 61: 459–464.

Nettles, W.C., 1990. *In vitro* rearing of parasitoids: role of host factors in nutrition. *Arch. Insect Bechem. Physiol.* 13: 167–175.

Obayashi, T., K. Iwabuchi and J. Mitsuhashi, 1994. *In vitro* rearing of a larval endoparasitoid, *Venturia canescens* (Gravenhorst) (Hymenoptera: Ichneumonidae). I. Embryonic development. *Appl. Entomol. Zool.* 29: 123–126.

Pair, S.D. and H.R. Gross, 1984. Field mortality of pupae of the fall armyworm, *Spodoptera frugiperda* (J. E. Smith), by predators and a newly discovered parasitoid, *Diapetimorpha introita*. *J. Georgia Entomol. Soc.* 19: 22–26.

Pair, S.D. and H.R. Gross, 1989. Seasonal incidence of fall armyworm (Lepidoptera: Noctuidae) pupal parasitism in corn by *Diapetimorpha introita* and *Cryptus albitarsis* (Hymenoptera: Ichneumonidae). *J. Entomol. Sci.* 81: 339–343.

Pair, S.D., 1995. Biology and rearing of *Diapetimorpha introita* (Cresson) (Hymenoptera: Ichneumonidae) on host and non-host noctuid pupae. *J. Entomol Sci.* 3: 468–480.

Pennacchio, F.S., B. Vinson and E. Tremblay, 1992. Preliminary results on *in vitro* rearing of the endoparasitoid *Cardiochiles nigriceps* from egg to second instar. *Entomol. Exper. Appl.* 64: 209–216.

Rojas, M.G., J.A. Morales-Ramos, E.G. King, G. Saldana and S.M. Greenberg, 1998. Use of a factitious host and supplemented adult diet to rear and induce oogenesis on *Catolaccus grandis* (Hymenoptera: Pteromalidae). *Environ. Entomol.* 27: 499–507.

Rotundo, G., R. Cavalloro and E. Tremblay, 1988. *In vitro* rearing of *Lysiphlebus fabarum* (Hym.: Braconidae). *Entomophaga* 33: 264–267.

Rice, J.W., N.B. Rankle, T.M. Gurganus, C.M. Marr, J.B. Barna, M.M. Walters and D.J. Burns, 1993. A comparison of large-scale Sf 9 insect cell growth and protein production: stirred vessel vs. airlift. *Biotechniques* 15: 1052–1059.

SAS Institute Inc., 1989. *SAS/STAT User's Guide, Version 6*, 4th Edn, Vol. 2. SAS Institute Inc., Cary, NC, 846 pp.

Thompson, S.N., 1979. Effect of dietary glucose on in vivo fatty acid metabolism and in vitro synthetase activity in the insect parasite, *Exeristes roborator* (Fabricius). *Insect Biochem.* 9: 645–651.

Trager, W., 1935. Cultivation of the virus of grasserie in silkworm tissue cultures. *J. Exp. Med.* 61: 501–513.

Vinson, S.B. and G.F. Iwantsch, 1980. Host regulation by insect parasitoids. *Q. Rev. Biol.* 55: 143–165.

Watanabe, M. and J. Mitsuhashi, 1995. *In vitro* rearing of an endoparasitic fly, *Exorista sorbillans* (Diptera: Tachinidae). *Applied Entomol. and Zool.* 30: 319–325.

Wyatt, S.S., 1956. Culture *in vitro* of tissue from the silk worm, *Bombyx mori* L. *J. Gen. Physiol.* 39: 841–852.

Yamamoto, Y., M. Ohori, T. Ohbayashi, K. Iwabuchi and J. Mitsuhashi, 1997. *In vitro* rearing of the larval endoparasitoid, *Venturia canescens* (Gravenhorst) (Hymenoptera: Ichneumonidae) II. Larval development. *Appl. Entomol. Zool.* 32: 256–258.

Addendum: Table 1. Chemical analyses of host pupae and diets: (1) Sf9Cell, (2) DI, (3) FBCell, (4) $FBControl_B$ and (5) Sf9 Control. Means are from 3 samples

	Diets					
	Pupae	1	2	3	4	5
Amino acids (µg/ml)						
Histidine	944.70	236.52	716.15	246.74	407.03	242.03
Asparagine	94.50	0.00	0.00	0.00	0.00	0.00
Arginine	618.26	0.00	0.00	181.36	200.59	0.00
Glutamine	2626.77	586.47	2702.13	239.50	219.98	408.02
Asp + ser	674.44	694.48	551.22	365.51	394.65	462.25
Glutamic acid	161.12	991.43	990.30	369.84	596.19	725.87
Threonine	985.59	490.73	0.00	0.00	0.00	0.00
Glycine	390.17	588.46	275.66	414.84	458.52	472.87
Alanine	2206.19	1022.09	616.09	653.88	540.39	582.67
Proline	705.60	445.94	448.69	282.85	327.59	303.37
Methionine	497.02	0.00	227.13	0.00	0.00	0.00
Valine	605.80	467.47	302.60	255.01	414.99	351.65
Phenylalanine	518.54	312.59	585.41	431.62	187.24	276.17
Isoleucine	1340.13	368.34	552.51	281.90	284.94	239.07
Leucine	796.57	1371.22	557.18	0.00	0.00	537.55
Lysine	2032.31	825.47	698.29	712.11	610.94	608.46
Tyrosine	919.07	1259.33	265.89	356.18	354.96	409.90
% Protein	10.83	6.9	4.4	4.7	4.9	5.0
Vitamins (µg/ml)						
Ascorbic acid	48.00	13.00	*	22.00	19.00	17.00
Pyridoxine HCl	178.67	4.00	*	5.00	3.00	5.00
Thiamine	23.00	0.00	*	0.00	0.00	0.00
Niacin amide	27.00	Traces	*	4.00	2.00	3.00
Folic acid	Traces	0.00	*	0.00	0.00	Traces
Riboflavin	1.73	75.00	*	Traces	Traces	143.00
B12	8.33	0.00	*	0.00	0.00	0.00
% Fatty acids						
Palmitoleic	0.120	0.502	*	0.170	0.022	0.484
Palmitic	0.924	4.264	*	0.956	0.461	4.034
Linolenic	0.289	2.428	*	0.227	0.076	2.066
Linoleic	0.306	6.525	*	0.587	0.193	6.278
Oleic	0.242	0.000	*	0.219	0.075	0.000
Steric	0.312	3.101	*	0.821	0.373	3.019
Anions (µg/ml)						
Fluoride	21.49	23.33	*	13.42	12.42	11.53
Chloride	114.53	350.93	*	464.86	382.78	278.56
Phosphate	830.43	196.66	*	96.18	103.11	89.39
Sulphate	14.65	74.32	*	84.19	68.63	38.25
Cations (µg/ml)						
Lithium	0.00	0.00	*	0.19	0.00	0.00
Sodium	10.65	69.86	*	11.08	54.21	54.70
Ammonium	20.09	28.44	*	8.07	19.01	24.68
Potassium	219.91	399.73	*	5.38	344.45	338.07
Magnesium	51.39	27.04	*	12.44	19.62	18.66
Calcium	6.29	9.73	*	10.24	9.53	6.67

* Data for diet 2 are unavailable.